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PATENT APPLICATION
Docket No. 15892.9

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of		)
	William Richard Cross et al.	)
Serial No.:	10/522,371	) Art Unit
Filed:	January 25, 2005	) 1657
Confirmation No.:	1386	)
For:	BIOMIMETIC UROTHELIUM	)
Examiner:	Laura J. Schuberg	)
Customer No.:	22913	)

DECLARATION OF JENNIFER SOUTHGATE, PH.D. AND WILLIAM CROSS, PH.D. UNDER 37 C.F.R. & 1.132

Mail Stop AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

We, Jennifer Southgate, Ph.D. and William Cross, Ph.D. hereby declare as follows:

1. We are both personally knowledgeable of the facts stated herein.

2. We are both co-inventors of U.S. Patent Application Serial No. 10/522,371 ("Subject Application").

3. Jennifer Southgate is an employee of The University of York and William Cross was a formal registered Ph.D. student of The University of York, which has ownership of the Subject

Application via assignment, and thereby we both have a personal interest in the Subject Application.

4. We both have significant experience in the art of human urothelium and the preparation of ex vivo human urothelium tissue and the stratification and differentiation thereof as applied in the Subject Application which is currently under examination. (*see* Appendix A: Curriculum Vitae of Jennifer Southgate, Ph.D; and Appendix B: Curriculum Vitae of William Cross, Ph.D.).

5. We have both reviewed and understand the Subject Application and the Cross and Zhang references. In fact, we both co-authored the Cross reference.

6. We have both reviewed the response to the Office Action being filed herewith, and attest that the properties of rat urothelium related to stratification and differentiation are different enough from human urothelium such that data based on rat urothelium cannot be directly applied to human urothelium.

7. We attest that with respect to the biological properties and responses to cell culture, rat urothelial cells are not the same as human urothelial cells. We will provide a wide range of evidence which shows that rat urothelial cells behave in the opposite way to human urothelial cells with respect to serum. This data shows any teachings from rat cells would not be applicable to human cells. Accordingly, it is surprising and unexpected that we were able to obtain stratified, terminally-differentiated human urothelium in which urothelial cells, isolated from the human body and propagated by culture in serum-free nutrient medium are transferred to a first nutrient differentiation medium containing serum and then redispersed by passage before being added to a second nutrient differentiation medium containing serum to form said urothelium.

8. We attest that expansion of urothelial cells, such as rat or porcine urothelial cells, does not equal differentiation or obtaining stratified, terminally-differentiated urothelium. Throughout the Office Action "culture" is used to cover all aspects of cell biology. All the papers cited in the Office Action are concerned with the expansion of urothelial cell numbers.

However, the Zhang reference does refer to differentiation, but there is no objective supporting evidence to show differentiation or stratification.

9. The claimed method is restricted to the differentiation and stratification of human urothelial cells into stratified, terminally-differentiated human urothelium, which is the polar opposite process to expansion of numbers of cells. Human urothelial cells cannot increase in numbers and functionally differentiate at the same time. Thus, anything taught about expansion of cells is not applicable to differentiation of human urothelial cells into stratified, terminally-differentiated human urothelium.

10. We attest that conditioned medium is not equivalent to bovine serum. Several of the references recited in the Office Action use conditioned medium to help the growth of urothelial cells. The conditioned medium is generated from rodent or human cell lines that require serum containing medium for growth. The feeder cells remove bovine factors and add different factors to create a hybrid medium, which can no longer be considered equivalent to medium containing serum. In fact, all of these papers show that the medium containing serum prior to conditioning has no useful effects, and thus, these references cannot be considered to teach the use of serum as advantageous to human urothelial cell stratification and terminal differentiation.

11. We attest that with respect to serum, human and rat urothelial cells respond in opposite fashions, and thus the Zhang paper, which only studies rat urothelium, is of no relevance to a method for preparing stratified, terminally-differentiated human urothelium. Any observation made on rat cells with respect to serum would not translate to, or teach anything about human urothelial cells (See Appendix C, Figure 1 for evidence).

12. We attest that the Ehmann paper (Appendix F) is of no relevance to a method for preparing a functional, differentiated human urothelium, as it teaches a method for prolonged expansion of porcine urothelial cells by co-culture with LA7 feeder cells in a nutrient medium that contains serum. This paper reports expression of tight junctions (ZO1) which is used to assume an "ionic barrier with transporting functions", but no functional evidence is given. As taught by Turner *et. al.* European Urology 54 (2008) 1423-1432. (Appendix G), porcine

urothelial cells are different from human cells in that they can show spontaneous expression of ZO1-containing tight junctions in culture but this does not indicate a functional urothelial barrier. Hence, it is not obvious from the Ehmann paper that transfer of urothelial cells propagated in serum-free nutrient medium to a first nutrient differentiation medium containing serum and then redispersal by passage before being added to a second nutrient differentiation medium containing serum would result in formation of a stratified differentiated urothelium.

13. The Office Action uses the term "culture" to cover both proliferation and differentiation of cells as if they are the same process when in fact they are polar opposite processes in the urothelium. Urothelial cells can be either proliferating or differentiated, but never both and to promote either one of these states requires very different conditions. This error is made clear on Page 9 Lines 4-6 of the Office Action where it is suggested the Cross reference refers to "establishing a primary culture with serum free media and expanding the cells in serum containing medium for subsequent passages" whereas the patent claims recite the opposite, that human urothelial cells are stratified and terminally differentiated by the claimed method steps. These steps do not expand human urothelial cells in serum.

14. The Zhang reference is largely concerned with proliferation of rat urothelium, which is the exact opposite of the claimed method which is solely concerned with differentiation. Although differentiation is mentioned in the title of the Zhang reference, no evidence of differentiation or function is given in the paper. The only marker used is CK17 which is expressed by all urothelial cells *in situ* as shown in Fig. 7a of the Zhang paper. In situ lower urothelial cell layers are undifferentiated whilst the upper layers show many markers of functional differentiation, however, none of such markers are shown to be present in the rat urothelial cells of the Zhang reference. The Zhang references focuses on the "long-term culture" aspect of the title, which is important as rat urothelial cells are still difficult to expand efficiently *in vitro*.

15. The Zhang paper teaches a method using conditioned-medium from mouse fibroblast cells to grow rat urothelial cells. By this process, mouse fibroblasts remove some bovine factors from the serum containing medium, and secrete different mouse factors into the medium, which

are then taken (with the medium) and used to enhance the proliferation of rat urothelial cells (Zhang, Fig. 2). The inclusion of components of bovine serum in this process is really a coincidental by-product of culturing mouse fibroblast cells which have to grow in medium with bovine serum, and as a result the conditioned-medium also contains some components of bovine serum. However, having some components of bovine serum does not constitute being "serum." The reason Zhang has components of serum in the urothelial cultures is a necessary by-product of the conditioned-medium, it is not used to elicit any specific effects. Furthermore, the Zhang paper explicitly states the serum is not useful for promoting rat urothelial cell growth or differentiation (see, pg. 427, col. 2, lines 5-8 of the discussion). Zhang states "[w]hen RUC were cultured in standard medium containing serum, the cells showed low plating efficiency, poor growth characteristics, a limited potential for cell division and failed to differentiate in vitro."

16. Conditioned-medium which originally contained serum cannot, once conditioned, be considered equivalent to a serum-containing medium. The serum has been transformed and is no longer serum. The mouse fibroblasts which perform the conditioning necessarily remove various bovine factors, and then add a plethora of their own factors; hence the wildly different results Zhang observed when comparing KSFM mixed with the conditioned medium to KSFM+DMEM (5%FBS).

17. There is no suggestion in Zhang that rat urothelial cells were ever passaged from (unconditioned) medium containing serum into another (unconditioned) medium containing serum. In fact, Zhang teaches that "...only in [conditioned-medium mixed with KSFM] did the cells achieve confluence." By contrast, "cells grown in other media only grew as a single layer without evidence of stratification and failed to reach confluence" (pg 427, col 1, lines 1-4). This shows that Zhang did not achieve stratified, terminally-differentiated urothelial cells. As a rule, if cells do not reach confluence they are not passaged, and since there is no direct mention of passaging cells in KSFM+DMEM (5%FBS), it is not possible for Zhang to teach anything related to passaging cells through serum. Thus, Zhang does not teach the method of producing stratified, terminally-differentiated urothelial cells which is presently claimed.

18. With further reference to Zhang, the reference teaches that the addition of serum to medium supports neither the growth nor differentiation of rat urothelial cells. Hence it is an inventive step and novel to find that human urothelial cells passaged through a first nutrient medium containing serum and then redispersed before being added to a second medium containing serum to form stratified, terminally-differentiated urothelial cells.

19. Whilst the expansion and differentiation of human urothelial cells can now be performed in defined media, the state of the art for rat urothelial cells still requires the use of conditioned medium and/or the direct contact of feeder cells. This recent paper (Appendix D; E.A. KURZROCK *et. al.* (2005). RAT UROTHELIUM: IMPROVED TECHNIQUES FOR SERIAL CULTIVATION, EXPANSION, FREEZING AND RECONSTITUTION ONTO ACELLULAR MATRIX, The Journal of Urology 173(1), 281-285) shows the current procedures for rat urothelial cell culture, and the striking differences in requirements between rat and human urothelia acts as further evidence that papers teaching techniques used on rat cells (e.g., Zhang) cannot be translated to human cells.

20. The Office Action cites Zhang at page 419 in the Materials and Methods section to teach growing RUC in KSFM + Serum. However, this section describes the isolation of RUC cultures specifically in "serum-free medium" and as a result is not relevant to producing stratified, terminally-differentiated human urothelium as presently claimed.

21. The Office Action cites Zhang at page 422, column 2 in the Materials and Methods section to teach passaging cells through serum. However, the rat urothelial cells were first established in serum free medium and then passaged into serum-containing medium, but never passaged from serum-containing medium into serum-containing medium. Furthermore it is stated in Zhang that "...only in [conditioned medium mixed with KSFM] did the cells achieve confluence. In contrast, cells grown in other media only grew as a single layer without evidence of stratification and failed to reach confluence" (pg 427, col 1, lines 1-4). As a rule, if cells do not reach confluence they are not passaged, and since there is no direct mention of passaging cells in KSFM+DMEM (5%FBS) this is not a correct assumption. This section of Zhang does

describe passaging of cells through KSFM + conditioned-medium, but this medium is not equivalent to serum-containing medium as shown by the results.

22. The Office Action cites Zhang at page 422, column 1, 3rd full paragraph and Table 1 media 6 to teach that KSFM + conditioned-medium contains serum when initially made up, it is therefore equivalent to serum-containing medium. This is an incorrect assumption. Conditioned-medium which originally contained serum cannot, once conditioned, be considered equivalent to serum-containing medium. The mouse fibroblasts which perform the conditioning will remove various factors of serum, and add a plethora of their own factors; hence the wildly different results Zhang observed when comparing KSFM mixed with the conditioned-medium to KSFM+DMEM (5%FBS).

23. The Office Action cites Zhang at page 422, column 1, to provide evidence of "expansion with different medias containing serum until passage 2, which would inherently require a second and third culture medium that includes serum." This section merely states growth curves were seeded at passage 2 at which point cells were put into each of the media (some containing serum). It also expressly states that cells were "detached and pooled and the cells were counted" showing the cells were not further cultured in serum. This section of Zhang is not evidence of passaging from one medium containing serum into a separate medium containing serum as suggested by the Office Action.

24. The Office Action cites Zhang at page 427 to teach that the final product of Zhang can be deemed to be the same as produced by the claimed method. This is untrue since although the Zhang paper claims to show differentiation, there were no objective markers used in the study, and thereby no evidence that the rat urothelial cells actually differentiated. The only marker used was CK17 which is expressed by all urothelial cells *in situ* as shown in Fig. 7a of the Zhang paper (in situ lower urothelial cell layers are undifferentiated whilst the upper layers show many markers of functional differentiation none of which are used in the Zhang paper and all of which can be seen when applying the claimed method to human urothelial cells).

25. The Office Action cites Zhang at page 427, column 1 to suggest that the claimed method must be missing a step to not produce the same results as achieved on rat tissue. The extra provided information (Appendix E) on the difference between rat and human urothelium demonstrate why the claimed invention is not missing a step.

26. The Office Action cites Zhang at page 428, column 1, paragraph 3 as evidence that serum supplemented medium has previously been used for long-term culture. This paragraph actually draws attention to the fact that rat urothelial cells are different to human urothelial cells in that they "cannot be maintained in KSFM alone long-term". Once again this citation assumes conditioned-medium and the serum-containing medium originally put onto the conditioning cells are equivalent which as previously stated is not true.

27. The Office Action cites Liebert at page 184, column 2, lines 22-32 to teach passaging urothelial cells through serum for the purposes of expansion. This part of the Liebert paper is actually citing a Chlapowski paper on the use of serum with rat urothelial cells for the purpose of expansion. Although not explicitly stated by Liebert (since they are just citing this study), the Chlapowski study is using conditioned-medium (just like the Zhang paper) from Swiss 3T3 cells which is radically different to serum-containing medium for reasons stated previously. Furthermore, in the Chlapowski study the use of Swiss 3T3 cell conditioned medium does prolong cell cultures and allowing their expansion, but expansion is the opposite of cell differentiation which is the aim of the claimed method (of which none is objectively shown).

28. The Office Action cites Freshney as evidence that people routinely passage cells through serum. Yes, they do routinely passage cells through serum in order to reduce cell density and maintain cell expansion. That is exactly why it is inventive to use passaging human urothelium cells in serum-containing medium to maintain or increase cell density whilst promoting a halt to cell expansion and functional differentiation; our claimed method achieves a result that is the exact opposite of what Freshney teaches all cell biologists. Therefore, the ability to obtain stratified, terminally-differentiated human urothelium is both surprising and unexpected under the teachings of Freshney.

29. We declare further that all statements made herein of our own knowledge are true and that all statements are made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed this 27th day of September, 2009.

Jennifer Southgate, Ph.D

William Cross, Ph.D

APPENDIX A

CURRICULUM VITAE for JENNIFER SOUTHGATE

Degrees

GIBiol	Biochemistry	2:1	Institute of Biology	1983
PhD			University of Leeds	1989

Posts held

Oct 1999 Professor of Molecular Carcinogenesis and Director of Jack Birch Research Unit, Department of Biology, University of York, York YO10 5YW, UK.

1992-99 Head of the Biology of Normal and Malignant Epithelial Cells Group, Imperial Cancer Research Fund Cancer Medicine Research Unit, University of Leeds, Leeds, UK.

1990-99 Research Fellow, then Senior, then Principal Research Fellow, University of Leeds, Leeds, UK.

1989-90 Research Officer, Imperial Cancer Research Fund, Clare Hall Laboratories, Hertfordshire, UK.

1978-89 Research Officer, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, UK.

Patents

W Cross and J Southgate. Biomimetic Urothelium (Patent Application GB0217314.4, EP03771199.1)

F Bolland, S Korossis, E Ingham, J Southgate. Improvements relating to decellularisation of tissue matrices for bladder implantation (UK Patent Application 0606231.9; U.S. Patent 2009-0130221 (pending))

Peer-Reviewed Publications from 2005

Cross WR, Eardley I, Leese HJ, Southgate J. A biomimetic tissue from cultured normal human urothelial cells: analysis of physiological function. Am J Physiol Renal Physiol 2005;289:F459-468.

Hall GD, Weeks RJ, Olsburgh J, Southgate J, Knowles MA, Selby PJ, Chester JD. Transcriptional control of the human urothelial-specific gene, uroplakin Ia. Biochim Biophys Acta 2005;1729:126-134.

Shaw NJ, Georgopoulos NT, Southgate J, Trejdosiewicz LK. Effects of loss of p53 and p16 function on life span and survival of human urothelial cells. Int J Cancer 2005;116:634-639.

Stahlschmidt J, Varley CL, Toogood G, Selby PJ, Southgate J. Urothelial differentiation in chronically urine-deprived bladders of patients with end-stage renal disease. Kidney Int 2005;68:1032-1040.

Varley C, Hill G, Pellegrin S, Shaw NJ, Selby PJ, Trejdosiewicz LK, Southgate J. Autocrine regulation of human urothelial cell proliferation and migration during regenerative responses in vitro. Exp Cell Res 2005;306:216-229.

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Crallan RA, Georgopoulos NT, Southgate J. Experimental models of human bladder carcinogenesis. Carcinogenesis 2006;27:374-381.

Garthwaite MA, Thomas DF, Subramaniam R, Stahlschmidt J, Eardley I, Southgate J. Urothelial differentiation in vesicoureteric reflux and other urological disorders of childhood: a comparative study. Eur Urol 2006;49:154-159, 159-160.

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Stransky N, Vallot C, Reyal F, Bernard-Pierrot I, de Medina SG, Segraves R, de Rycke Y, Elvin P, Cassidy A, Spraggon C, Graham A, Southgate J, Asselain B, Allory Y, Abbou CC, Albertson DG, Thiery JP, Chopin DK, Pinkel D, Radvanyi F. Regional copy number-independent deregulation of transcription in cancer. Nat Genet 2006;38:1386-1396.
Varley CL, Garthwaite MA, Cross W, Hinley J, Trejdosiewicz LK, Southgate J. PPARgamma-regulated tight junction development during human urothelial cytodifferentiation. J Cell Physiol 2006;208:407-417.
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Bolland F, Turner AM, Southgate J. Concepts and Progress in Bladder Tissue-engineering. Engineering in Medicine 2008.
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Shabir S, Southgate J. Calcium signalling in wound-responsive normal human urothelial cell monolayers. Cell Calcium 2008; 44:453-64.
Turner AM, Subramaniam R, Thomas DF, Southgate J. Generation of a Functional, Differentiated Porcine Urothelial Tissue In Vitro. Eur Urol 2008; 54:1423-32.
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Chopra B, Georgopoulos NT, Nicholl A, Hinley J, Oleksiewicz MB, Southgate J. Structurally diverse

APPENDIX B

Curriculum Vitae

William Cross

Personal Details

Name William Richard Cross

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Qualifications

B. Med. Sci.	University of Nottingham	1993
B.M. B.S.	University of Nottingham	1995
MRCS	The Royal College of Surgeons of England	1998
PhD	University of York	2004
FRCS (Urol)	The Royal College of Surgeons of England	2007

Current Appointment

Consultant Urological Surgeon
Pyrah Department of Urology
St James's University Hospital
Leeds
LS9 7TF

Past Appointments

Oct 2002 – June 2008	Urology Specialist Registrar	Yorkshire Deanery
Oct 1999 – Sept 2002	PhD Research Fellow	University of York
Aug 1997 – Sept 1999	Basic Surgical Trainee	Leeds General Infirmary
Feb 1997 – July 1997	Anatomy Tutor	University of Leeds
Aug 1996 – Jan 1997	A/E SHO	St James's University Hospital
Feb 1996 – July 1996	Surgical HO	University Hospital, Nottingham
Aug 1995 – Jan 1996	Medical HO	York Hospital

Courses

Management and leadership development	Leeds	2007-8
Making the transition to consultant	Leeds	2007
FRCS(Urol) revision course	Oxford	2007
European Urology Residents Education Programme	Prague	2006
Laparoscopic surgery wet lab	Paris	2005
Statistics for clinical trials	York	2005
International live radical pelvic surgery master class	Leeds	2005
Urodynamics	Bristol	2005
Communication skills for urologists	Leeds	2004
Urology in spinal injuries	Sheffield	2003
Scientific basis of urology	London	2003
Radiation protection of patients and staff	Leeds	1999
Intermediate laparoscopic surgery	Leeds	1998

Prizes and Awards

European Urological Scholarship - Clinical Fellowship (€3000) 2007
Bert Inman Research Fellowship (£58968) 2004-2007
European Association of Urology award for best paper published in urological literature on fundamental research (€5000) 2005
Yorkshire Urology Annual Audit Meeting Prize 2005, 2004 and 2001
Patented technique for urothelial cell culture (GB0217314.4, EP03771199.1)
Urological Research Society Annual Paper Prize (£3000) 2004
The British Urological Foundation/Wyeth Research Scholarship 2000-2001
The Ralph Shackman Trust Research Fellowship 2000-2001

Publications

Book Chapters

Treatment of pain in urology
AD Joyce and WR Cross
Drug treatment in urology (Blackwell Science 2006)

Chapter 2: Perioperative management
WR Cross and I Ahmed
MRCS Core Modules: Essential Revision Notes (Pastest 1999)

Peer Reviewed Articles

PPARγ-regulated tight junction development during human urothelial cytodifferentiation. CL Varley, MAE Garthwaite, WR Cross, J Hinley, LK Trejdosiewicz and J Southgate. J Cell Physiol. 2006 Aug; 208(2): 407-17

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Tissue engineering and stem cell research in urology. WR Cross, DFM Thomas and J Southgate. BJU International. European Urology Update Series 2003; 92: 165-171

Bladder reconstruction – from cells to materials. J Southgate, WR Cross, I Eardley, DFM Thomas and LK Trejdosiewicz. Proc Inst Mech Eng [H]. 2003; 217(4): 311-6

The current use of biomaterials in urology. SN Lloyd and WR Cross. Eur Urol. Supplements 2002; 1: 2-6

The efficacy of a range of contact media as coupling agents in extracorporeal shockwave lithotripsy. JJ Cartledge, WR Cross, SN Lloyd and AD Joyce. BJU International 2001; 88: 321-324

Hydroureteronephrosis secondary to diverticular abscess. WR Cross, R Wilson and I Eardley. Surgery 2000 June; 18(6): 152a

Traumatic pseudoaneurysm of the superficial temporal artery. WR Cross and H Nishikawa. Journal of Accident and Emergency Medicine 1999 Jan; 16(1): 73

APPENDIX C

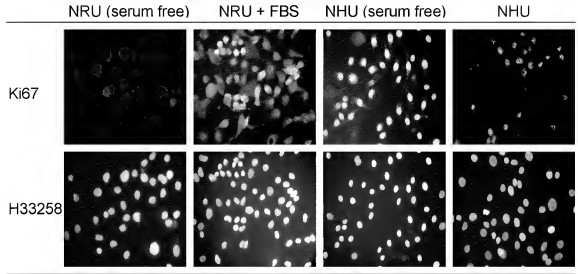

Fig. 1 – In culture Ki67 can be used as a proliferation marker for urothelial cells. Normal rat urothelial cells (NRU) show low proliferation in serum-free medium but higher levels in serum whereas normal human urothelial cells show the opposite; reduced proliferation in serum. H33258 is a nuclear stain which shows the total number of cells in each field of view.

APPENDIX D

Application No. 10/522,371
Declaration of Jennifer Southgate, Ph.D. and William Cross, Ph.D.

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RAT UROTHELIUM: IMPROVED TECHNIQUES FOR SERIAL CULTIVATION, EXPANSION, FREEZING AND RECONSTITUTION ONTO ACELLULAR MATRIX

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ABSTRACT

Purpose: The rat has been a cost-effective model for the evaluation of bladder development, cancer and stromal-epithelial interactions. Serial cultivation of rat urothelium has been difficult. We developed a reliable protocol for the harvest, serial cultivation and cryopreservation of rat urothelium. We investigated the differentiation markers of in vivo bladder urothelium compared with cells reconstituted onto an acellular bladder matrix.

Materials and Methods: Epithelial harvest techniques using trypsin and collagenase were compared. Medium and conditions were optimized for serial culture and growth characteristics were calculated. Cultured cells were cryopreserved, and then recovered and grown on acellular bladder matrices. Morphology and markers of differentiation were compared between normal bladder and engineered grafts using scanning electron microscopy (SEM) and immunohistochemistry.

Results: Atraumatic enzymatic removal of urothelium with trypsin yielded more cells with greater viability than collagenase. Cells could be reliably grown beyond 10 passages using fibroblast conditioned medium and a 3T3 feeder layer during initial passages. Cryopreserved cells were successfully recovered and incorporated onto acellular matrices. Immunostaining and SEM of engineered grafts demonstrated early markers of differentiation, such as surface microvilli and cytokeratin 17, on polygonal cells with typical tight junctions.

Conclusions: Rat urothelium can be reliably grown using fibroblast conditioned medium and a 3T3 feeder layer during primary culture. Serially passaged cells can survive cryopreservation and they are able to reconstitute epithelium on an acellular bladder matrix. Cells that are incorporated into the matrix express markers of early differentiation and demonstrate typical morphological characteristics by SEM. These culture techniques and this in vitro organoid model should facilitate the use of rat urothelium.

KEY WORDS: urothelium, rats, Sprague-Dawley, bladder, tissue engineering, extracellular matrix

The rat has been a cost-effective model for the evaluation of bladder development, inflammation, cancer, gene therapy and stromal epithelial interactions.[1,2] Unlike in larger mammals, a reliable technique for the harvest, cultivation, expansion and reconstitution of rat bladder has not been described. In the last 2 decades great strides have been made in our ability to passage rat urothelium with modifications of culture conditions and growth factors.[3–6] Until recently serial cultivation of these cells beyond 5 passages has been difficult due to early senescence.

The urothelium of large mammals can be separated from the underlying stroma without great difficulty. In mice and rats urothelial harvest is tedious, difficult and almost universally accompanied by fibroblast contamination. Rozzell et al introduced a novel method of rat urothelial harvest via bladder eversion.[7] Our first goal was to modify this technique and develop a reliable protocol for long-term rat urothelial culture. Our second goal was to determine if these cells could be frozen for long-term storage. Our third goal was to characterize differentiation markers of adult rat urothelium by immunohistochemistry (IHC) and scanning electron microscopy (SEM).

Acellular rat bladder matrix has been used for bladder augmentation and the evaluation of mesenchymal-epithelial interactions.[1,2,8] Epithelial lined tissues from cultured rat urothelium have been constructed in vivo.[9,10] On the other hand, to our knowledge in vitro bioengineering of a urothelial graft for bladder wall replacement in small animals has not been reported. Our fourth goal was to construct in vitro a urothelial lined graft on acellular bladder matrix to evaluate epithelial differentiation. Unlike synthetic gels and membranes, the growth of urothelium on matrix may better replicate the bladder. This organoid model will facilitate the study of urothelial differentiation, tight junctions and stromal-epithelial interactions.

MATERIALS AND METHODS

Animal experiments were performed in accordance with the Animal Use and Care Committee at University of California. Bladders were obtained from Sprague-Dawley rats weighing 200 to 550 gm. The bladder was inverted by pushing the dome downward through the bladder neck with a blunt 18 gauge needle (fig. 1, A). A suture was tied around the neck and tightened with a half knot after the needle was inserted. The inverted bladder was inflated with phosphate

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Application No. 10/522,371
Declaration of Jennifer Southgate, Ph.D. and William Cross, Ph.D.

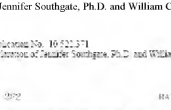

FIG. 1. A, blunt needle inserting rat bladder. B, inflation of fixated bladder.

buffered saline (PBS) through the needle (fig. 1, B). As the needle was removed, the suture was tightened, producing a dissected ball with only the urothelial surface exposed.

Urothelial harvest. To determine the most efficacious technique for epithelial removal bladders from 8 age and weight matched animals were evaluated with 2 enzyme regimens, namely trypsin - 0.05% ethylenediaminetetraacetic acid (EDTA) (0.53 mM) and collagenase type IV (CLS4, Worthington Biochemicals, Lakewood, New Jersey). Inverted bladders were pinced in 25 ml of either solution in a spinner flask and stirred in a 5%/75% CO_2 cell culture incubator for 1 hour. After 1 hour bladders were placed in a 60 mm Petri dish containing 5 ml fibroblast medium (FM), consisting of Dulbecco's modified Eagle's medium supplemented with 2.92 g/ml L-glutamine (Cellco, Grand Island, New York) and 10% fetal bovine serum (FBS) (Omega Scientific, Tarzana, California), and 1% antibiotic-antimycotic solution, containing of penicillin (600 U/ml), streptomycin (600 μg/ml), gentamicin sulphate (1.25 μg/ml), and fungizone (2.5 μg/ml) (HyClone). Bladders were gently scraped with a scalpel blade and removed from the cell suspension. All cell separation from the spinner flask and the 60 mm Petri dish was transferred to 50 ml centrifuge tubes. An equal volume of FM was added to each tube to neutralize trypsin and cells were centrifuged at 1,000 rpm for 5 minutes. Supernatant was removed and cells were pooled after resuspension in a supported keratinocyte growth medium (KGM)—, consisting of keratinocyte basal medium supplemented with bovine pituitary extract (60 μg/ml), hydrocortisone (0.5 μg/ml), insulin (5 μg/ml), epidermal growth factor (0.1 ng/ml), gentamicin (50 μg/ml), amphotericin (50 ng/ml) (BioWhittaker, Walkersville, Maryland), dilution recombinant cholera toxin (8.3 ng/ml) (Calbiochem, San Diego, California) and 2% FBS. Cells were counted in a hemocytometer chamber and viability was evaluated by trypan blue staining. Differences between the 2 regimens were evaluated with Student's t test.

Cell culture. Our initial study of rat urothelium demonstrated superior growth of cells on 3T3 feeder layers. This led to the inhibition of confluent cell monolayers from 3T3 cell cultures. After cell harvest cell suspensions were spun down and then resuspended in a 1:1 mixture of fibroblast conditioned growth medium (FCM) and KGM—. Cells were seeded onto 3T3 layered plates for selective proliferation of urothelium. Plates were incubated in a humidified atmosphere of 5% CO_2 at 37C. Medium was changed every 2 to 3 days. The 1:1 mixture of medium was changed to KGM— alone by the second or third medium change. The use of 3T3 feeder cells was discontinued after the second passage. Total cell numbers (growth curves) were determined after 2 subsequent passages (p4) for 10 different strains. For this assay urothelial cells were resuspended in KGM+ and plated in a 24-well tissue culture plate at a density of $5 \cdot 10^4$ cells per well. Medium was changed every 2 to 3 days. Every 3 days cells in 3 wells were detached with 0.25% trypsin-1 mM EDTA and counted as described.

3T3 feeder cell preparation. Murine 3T3 cells (~8 passages) were grown to confluence in FM. Confluent cultures of 3T3 cells containing approximately $4 \cdot 10^7$ cells per 150 mm plate were treated for 1 hour with 0.01 mg/ml mitomycin C in a 5%/75% O_2 cell culture incubator. Medium containing mitomycin C was then aspirated and cells were rinsed 3 times with PBS. Cells were then trypsinized with 0.25% trypsin-1 mM EDTA for 10 minutes, resuspended with an equal volume of FM and centrifuged at 1,000 rpm for 5 minutes. Supernatant was removed and cells were resuspended in FM. Cells were plated into 60 mm cell culture dishes at a density of about $5 - 10^5$ cells per dish and allowed to adhere to the dishes for at least 1 hour. The FM on was aspirated and the dishes were rinsed 2 times with FM before urothelial cells were plated.

FGM preparation. Murine 3T3 cells were grown to confluence in growth medium (GM) with medium changes every 3 to 4 days. GM consisted of Dulbecco's modified Eagle's medium supplemented with epidermal growth factor (0 ng/ml), cholera toxin (8.3 ng/ml), hydrocortisone (1 μg/ml), ARAM (5%), L-glutamine (2.92 μg/ml) and 10% FBS. At each medium change spent fibroblast conditioned medium (FGM) was collected and filtered through a 0.22 μm filter and stored at 4C. When the last aliquot of FGM was collected at 3T3 confluence, all stored aliquots were pooled and filtered a second time through a 0.22 μm filter. FGM was then stored in small aliquots at −20C. FGM was thawed only once before use and stored at 4C thereafter.

Cryopreservation. Cells were removed from tissue culture dish with 0.25% trypsin-1 mM EDTA and an equal volume of FM was added to the cell suspension before centrifugation. Supernatant was aspirated and cells were resuspended in 90% KGM+, 40% FBS and 10% dimethyl sulfoxide (Sigma Chemical Co. St. Louis, Missouri). Aliquots (1 ml) were placed in cryopreservation vials. Cells were frozen at a rate of 1C per minute in the liquid nitrogen vapor of a 3MVIC cryogenic biological freezer (Taylor-Wharton, Theodore, Alabama) and then transferred to the liquid phase where frozen. Cells from 10 strains were thawed and growth characteristics determined as described.

TRITC-SEM. Whole bladders were excised, fixed in Streck tissue fixative (Streck Laboratories, La Vista, Nebraska) processed, sectioned and stained with hematoxylin and umbelliferone as described previously [7]. Antibodies against cytokeratin were evaluated, including cytokeratin (CK)8 and 14, CK17 (Sigma Chemical Co.), CK20 and pancytokeratin (Novocastra, Newcastle, United Kingdom) and uroplakin III (Santa Cruz Biotechnology, Santa Cruz, California). Two bladders were fixed in modified Karnovsky's fixative for SEM. They were evaluated with a Philips XL30 ESEM-FEG Company, Hillsboro, Oregon, as described previously [7].

Tissue culture. Bladder acellular matrices (BAM) were prepared from rat bladders using a modified method described by Piechota et al. Whole bladders were expanded and rinsed in PBS with 0.1% sodium azide. Bladders were cut along 1 side to expose the epithelial layer and the mucosa was scraped off with glass slides. Bladder pieces were then placed in a spinner flask in PBS/0.1% sodium azide and stirred at room temperature for 5 to 6 hours. Bladder pieces were rinsed in PBS and stirred 6 to 8 hours at room temperature in 1 M sodium chloride with 2,000 Kunitz U deoxyribonuclease I (Sigma Chemical Co.). Bladder pieces were rinsed in PBS and then stirred for 4 hours at room temperature in 4% hydrated 1% sodium azide. This step was repeated once with fresh reagent. Matrices were washed 2 times in PBS and stored at 4C in PBS with 10% ABAM. To determine if there were cells remaining 3 BAMs were fixed in STF, processed, sectioned and stained with hematoxylin and umbelliferone against pancytokeratin. Another BAM was evaluated with SEM.

For bioengineered bladder tissue frozen rat urothelial cells were thawed and plated onto 100 mm 3rd culture dishes in KGM—. When cultures were 60% to 70% confluent, cells were

Application No. 10/522,371
Declaration of Jennifer Southgate, Ph.D. and William Cross, Ph.D.

trypsin and pelleted by centrifugation, and resuspended in 50 to 100 μl RGM+. The resultant cell suspension was seeded onto four 2 pieces of HAM in an organ culture dish. Ten replicate grafts were grown. Cells were allowed to adhere for 2 to 3 hours and then 2 ml RGM+ were gently added to each dish. One HAM that served as a negative control was cultured without cells. Medium was changed every 2 to 3 days. After 3 days to 1 week medium was changed to GM. After 4 weeks in culture, grafts were rinsed with PBS and fixed in Bouin's fixative for IHC or in modified Karnovsky's fixative for SEM.

RESULTS

Urothelial harvest. There were significant differences in cell yield and viability between the 2 enzyme protocols in 8 bladders each (p < 0.05). The protocol with trypsin removed more cells than that with collagenase (1.43×10^6 vs 0.87×10^6 cells/ml with greater viability 98% vs 79%) (fig. 2).

Urothelial culture. With the described protocol 9 cell strains were grown beyond 10 passages. For the initial selection, growth of urothelium 3T3 feeder layers were critical. After the second passage a feeder layer was not necessary. Although FCM was not required, it enhanced the primary culture. More than 10 strains of cultured cells were frozen. All demonstrated the ability to proliferate after thawing. Comparison of growth curves for the same passage (p4) before and after cryopreservation did not demonstrate a significant difference (fig. 3).

IHC/SEM of whole bladder. Intact, whole bladder sections demonstrated characteristic transitional epithelium that varied from 1 to 4 cell layers. Immunostaining against uroplakin, CK8 and CK20 showed typical staining of superficial cells and the apical cells. CK14 localized to the basal layer and CK17 expressed in basal and intermediate cells (see table, fig. 4, A and D). hIM revealed ridges on the surface of polygonal apical cells with well-formed tight junctions between the cells (fig. 4, C and D). Cells varied between 30 and 50 μm in diameter.

Urothelial differentiation by immunohistochemical staining for CK's and uroplakin III in rat bladder and engineered grafts.

Differentiation Marker	Rat Bladder Intact	Engineered Graft
CK+	Apical	Yes
CK13	none	Yes
CK17	Basal/Intermediate cells	Yes
CK20	superficial cells	Yes
Uroplakin III	Superficial cells	No

Tissue culture. As prepared, bladder acellular matrices were found to be free of epithelial cells by hematoxylin and acetylcholinesterase staining (fig. 5, A). SEM demonstrated the epithelium-collagen fibrils and extracellular matrix without urothelial cells (fig. 5, B). The negative control graft, that is matrix grown without cells, had no evidence of epithelial cells.

Engineered grafts showed a monolayer of epithelial cells covering the majority of the exposed luminal surface. In some areas there was stratification with 2-cell layers. The epithelium stained positive for pancytokeratin, CK8, CK13 and CK17 but not uroplakin III or CK20 (see table, fig. 6, A and B). SEM demonstrated microvilli thoroughly covering the apical cells (fig. 6, C to E). Cells were 10 to 30 μm in diameter and polygonal with well-defined tight junctions.

DISCUSSION

The rat is a useful model for urological studies. They are relatively inexpensive, small and widely available for tissue sharing. Despite their size they are large enough for reconstructive surgery and urodynamic testing.[7–9] Despite its popular use in urological studies serial cultivation of rat urothelium beyond 5 passages has been difficult due to early senescence.[7,10] At our laboratory despite ample experience culturing keratinocytes and cervical epithelial cells we nevertheless had initial difficulty serially cultivating rat urothelium.

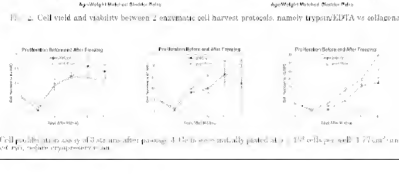

FIG. 2. Cell yield and viability between 2 enzymatic cell harvest protocols, namely trypsin/EDTA vs collagenase.

FIG. 3. Cell proliferation assay of 3 rat urothelial passages. Cells were initially plated at 1×10^5 cells per well/1.77 cm² and counted every 2 days. Pre-Cryo, before cryopreservation.

Application No. 10/522,371
Declaration of Jennifer Southgate, Ph.D. and William Cross, Ph.D.

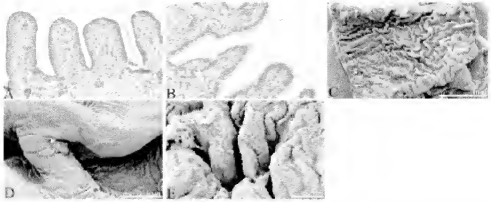

FIG. 1. Rat bladder images. A, trypsin/EDTA staining, reduced from ×20. B, CK17 IHC staining, reduced from ×20. C, bulk scanning electron micrograph of luminal surface, overall edges showing polygonal cells.

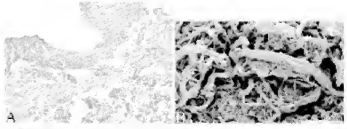

FIG. 2. Acellular matrix made from rat bladders. A, no epithelial cells. IHC staining for pancytokeratin, reduced from ×20. B, scanning electron micrograph shows cytoskeleton and extracellular matrix.

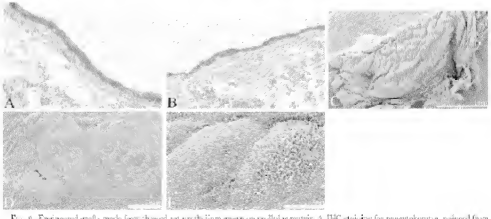

FIG. 6. Engineered grafts made from cultured rat urothelium grown on acellular matrix. A, IHC staining for pancytokeratin, reduced from ×40. B, IHC staining for CK17, reduced from ×20. C to E, scanning electron micrographs of luminal surface, overall urothelial showing polygonal cells.

By modifying the harvest technique of Rossell et al. we found that trypsin efficiently removed the urothelium with excellent cell viability. We found that, although a TCI feeder layer as well as fibroblast conditioned medium were not requisite, they enhanced the primary culture and minimized fibroblast contamination. KSFM + was sufficient for subse-

Application No. 10/522,371
Declaration of Jennifer Southgate, Ph.D. and William Cross, Ph.D.

quent passages and the feeder layer was not necessary after the second passage. Rat urothelial cells can be routinely grown in the long term and cryopreserved for later use.

We found that CK expression followed the pattern noted by other groups with CK5/14 basal weighted, CK8, 18 and 20 luminal, and CK17 appearing in basal and intermediate cells.[13, 14] Our SEM images of normal bladder are consistent with those in other studies, which showed polygonal cells (40 to 70 μm) with distinct tight junctions.[15] Superficial cells were covered with ridges. Asymmetrical unit membrane, which is made up of uroplakins, gives urothelium this unique appearance.[16]

Uroplakin is unique to the differentiated surface cells. Immature cells, apical or not, do not express uroplakin. Several investigators have noted this by treating rats with cyclophosphamide, protamine sulfate or saccharin, which denudes the bladder. During early regeneration of the urothelium immature apical cells appeared smaller, and they are covered with microvilli and lack uroplakin. Eventually the microvilli disappear or possibly coalesce and the ridge-like architecture develops with concurrent uroplakin expression.[13, 15]

Cytokeratin expression also changes with regeneration. CK17 appears in all layers during early regeneration, eventually localizing to the intermediate and basal layers. CK20 is not expressed during early regeneration (differentiation). When CK17 expression ceases in superficial cells, CK20 synthesis starts.[13]

Mesenchymal and epithelial development and interaction in vivo have been extensively described by Baskin et al.[14] One of our goals was to develop an in vitro model for the further study of rat urothelium. Rather than using a synthetic gel,[11] we chose to use bladder acellular matrix for a better replication of the extracellular environment. We were able to efficiently remove cells from bladder specimens without interfering with extracellular matrix ability to support epithelial growth. Urothelial cells that incorporated the matrix assumed a surface morphology resembling regenerating rat bladder. The cells are small (10 to 30 μm), carpeted with microvilli and lack uroplakin. CK17 is demonstrated but not CK20. We hypothesize that while the matrix is able to facilitate cell growth, it is unable to induce terminal differentiation.[11]

Differentiation of urothelium depends on culture conditions and the substrate. Howlett et al observed terminal differentiation of rat urothelium when plated on collagen gels incorporated with 3T3 cells.[17] On the other hand, gels without stroma only supported the growth of immature cells. The same group found that calcium induces the stratification and differentiation of human urothelium. Calcium alone without stroma was insufficient to induce terminal differentiation based on uroplakin and integrin expression.[18] On the other hand, more recent studies by others has shown the ability of human and rabbit urothelium to achieve almost terminal differentiation without the presence of stroma.[19, 20] In the 2 studies cells expressed uroplakins and had permeability coefficients similar to those of normal bladder.

CONCLUSIONS

The rat is a cost-effective model for the evaluation of urothelial biology. More than 1 million cells per bladder can be removed atraumatically with trypsin. Rat urothelium can be reliably grown using fibroblast conditioned medium and a 3T3 feeder layer during primary culture. Serially passaged cells can survive cryopreservation and are able to reconstitute an acellular bladder matrix. Cells that incorporate the matrix express early markers of differentiation and demonstrate typical morphological characteristics by SEM. These culture techniques and this in vitro organoid model should facilitate the use of rat urothelium.

Murine 3T3 cells were provided by Dr. Robert H. Rice, University of California-Davis. CK5 and 14 were provided by Declan Lane, University of Dundee, Dundee, United Kingdom. Grete Adamson and Xuqun Feng assisted with SEM.

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APPENDIX E

The following information is extracted from a manuscript currently in preparation on comparing human and rat urothelial cell culture systems.

Abstract

Normal rat urothelial cell cultures were established using a comparable method used to culture normal human urothelial cells, thus providing a platform for cross-species in vitro comparisons. Differences in proliferation and differentiation of rat and human urothelial cells were observed.

Materials and Methods

Tissues and Cell Culture

Normal human urothelium

The collection of surgical specimens was approved by the relevant Local Research Ethics Committees and had full informed patient consent. Surgical specimens of normal urothelium (ureteric) were obtained from patients with no histological evidence of urothelial dysplasia or malignancy. Tissues were collected in transport medium containing HBSS with HEPES (10mM; pH 7.6) and aprotinin (20 KIU; Trasylol, Bayer plc, Newbury, UK) and were used to establish finite normal human urothelial (NHU) cell lines, as described previously (1, 2).

Normal rat urothelium

Adult male Sprague Dawley rats (225-250g; Charles River Ltd, UK) were euthanized in accordance with UK Home Office Schedule 1. Thereafter, urinary bladders were rapidly excised and collected into ice-cold transport medium. Bladders were dissected into smaller pieces and incubated for 4 hours at 37°C in 'stripping solution' containing Ca^{2+}/Mg^{2+}-free transport medium and 0.1% w/v EDTA. The urothelium was then gently separated as intact sheets from the underlying stroma and collected by centrifugation. Following resuspension, urothelial sheets were incubated in 200 U/ml collagenase type IV (Sigma-Aldrich) for 20 minutes at 37°C and disaggregated by gentle pipetting. Normal rat urothelial (NRU) cells pooled from three rats were seeded into 25cm^2 Primaria® tissue culture flasks (Falcon) in growth medium overnight.

Maintenance of NHU and NRU cell cultures

NHU and NRU cell cultures were maintained in Primaria® tissue culture flasks in KSFMc medium, consisting of keratinocyte serum free medium (KSFM) supplemented with bovine pituitary extract (BPE) and epidermal growth factor (EGF) at the manufacturer's recommended concentrations (Invitrogen, Paisley, UK), and cholera toxin (30ng/ml; Sigma-Aldrich){Southgate, 1994 #32; Southgate, 2002 #31}. NHU cell lines were harvested for subculture by incubation for 5 min in PBS containing 0.1% (w/v) EDTA, followed by minimum incubation with 0.25% (w/v) trypsin in 0.02% (w/v) EDTA to detach cells and collection into medium containing 1mg.ml^{-1} trypsin inhibitor (SigmaAldrich, Poole, UK). NHU cells were grown to sub-confluence (80-90%) in 25cm^2 tissue culture Primaria® flasks (BD Biosciences, Oxford, U.K). NRU cell cultures were used between passages 1-2 and NHU cells were used between passages 2-5.

NHU cells were differentiated as described by Cross et al (11). Briefly, NHU cell cultures were grown to confluence, thereafter, the growth medium was supplemented with 5% fetal bovine serum (FBS) and 2mM calcium chloride, to stimulate differentiation. NHU cells were maintained in differentiation medium for a period of up to 28 days. A similar protocol was used to treat NRU cultures, with the exception that following preliminary investigations, the calcium concentration of the serum-supplemented medium was not modified.

Indirect Immunofluorescence microscopy

Cultured human or rat urothelial cells were grown to 70-80% confluence on 12 well glass slides in KSFMc ± 5% FBS and fixed using methanol:acetone (1:1) solution for 5 minutes. Slides were then air-dried and incubated overnight at 4°C with primary antibodies. Following several washes in PBS, bound antibodies were detected using either goat anti- mouse (1:700) or anti-rabbit (1:400) immunoglobulins conjugated to Alexa Fluor® 488 (Invitrogen, Paisley, U.K.). Omission of primary antibodies was used as a negative control. Hoechst 33258 (0.1μg/ml; Sigma-Aldrich) was added to the final wash to visualise nuclei. Slides were examined under epifluorescence illumination on an Olympus BX60 microscope.

Results

NRU cell culture and characterization

Cultures of NRU cells were successfully established using the same isolation and maintenance procedure used for NHU cells (Southgate ref). However, the attachment of primary NRU cells was less efficient than observed with NHU cells, with up to 50% of cells failing to adhere to the flasks following overnight seeding. In culture, NRU cells exhibited an epithelioid morphology and grew mainly in colonies (Figure 1). The rate of NRU cell proliferation was significantly slower than NHU cells. NRU cultures also had a shorter lifespan, which beyond passage two underwent widespread cell death. This was in contrast with NHU cell cultures, which exhibited a higher plating efficiency (>90%), grew as dispersed monolayers in sub-confluent culture and were sustained up to six passages, consistent with previous studies (1, 2).

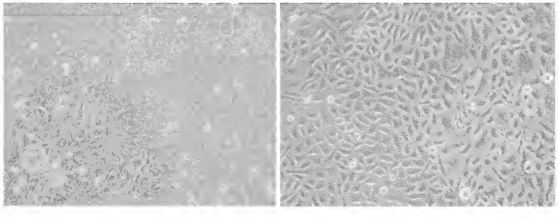


Figure 1. Photomicrographs showing morphology of rat (A) and human (B) urothelial cells in culture.

In the presence of 5% FBS, an increased rate of NRU cell proliferation was observable compared to serum-free cultures, as assessed by [^{3}H]-thymidine incorporation. The number of NRU cells in the absence of FBS was 0.80 x 10^6 cells/ml compared to 1.51 x10^6 cells with FBS (n=3) at first passage. These findings were further supported by immunofluorescence analysis of Ki67 antigen expression, which was negative in NRU cells grown in KSFMc, but in equivalent cultures grown in 5% FBS, clusters of Ki67$^+$ cells were present and also surrounded by progressively less intense Ki67$^+$ cells, suggesting a differentially proliferating or progenitor sub-population (Figure 2). These observations contrasted with NHU cell cultures, which were positive for Ki67 in serum free medium, but exhibited a reduction in the proportion of Ki67$^+$ cells in the presence of 5% FBS (Figure 2).

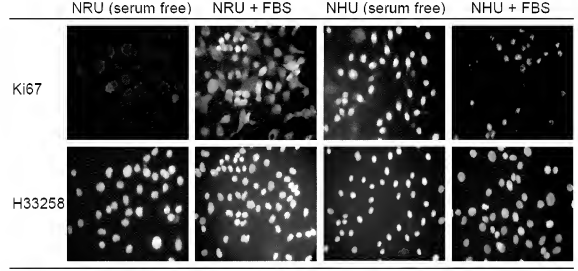
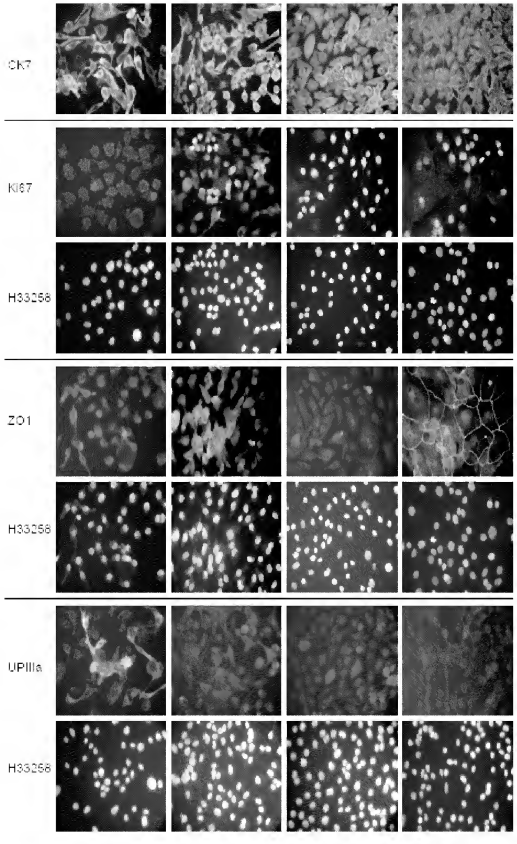


Fig.2 – Immunofluorescence labeling of Ki67, a cell cycle stage marker, in normal rat urothelial (NRU) and normal human urothelial (NHU) cells in the presence and absence of fetal bovine serum (FBS). These micrographs show the expression levels of Ki67 are always the complete opposite in NHU and NRU cells. H33258 is a DNA intercalating dye used to illustrate total cell number in a field of view.

Indirect immunofluorescence revealed that both NRU and NHU cell cultures were positive for CK7 and there was no detectable contamination by smooth muscle actin-positive stromal cells. NRU cells showed intercellular expression of the tight junction-associated proteins (e.g. ZO-1), irrespective of the presence of FBS in the medium. However, in the presence of 5% FBS, the expression of tight-junction-associated proteins, including ZO-1 were increased and were translocated to intercellular borders. UPIIIa expression was not detected in NHU or NRU cell cultures grown with FBS, but was detected in a majority of NRU cells grown in the absence of FBS (Figure 3). Figure 3 shows the immunofluorescence of cultures of Normal Rat Urothelium cells in serum-free medium (column 1), Normal Rat Urothelial cells with serum (column 2), Normal Human Urothelial cells in serum-free medium (column 3), and Normal Human Urothelial cells in medium with serum (column 4).


CK7
Ki67
H33258
ZO1
H33258
UPIIIa
H33258

Discussion

As part of the bridging between in vivo to in vitro and rat to human studies, we have transferred our established methodologies for the isolation and in vitro propagation of NHU cells to NRU cells, providing an experimental platform for in vitro cross-species comparisons. In both preparations, the urothelium was first separated from the underlying tissue to allow efficient harvesting of urothelial cells with minimal stromal contamination and the same culture conditions were applied. However, there were fundamental differences between NRU and NHU cell cultures, including a low initial plating efficiency and reduced proliferation capacity for the former. Of particular note was the effect of FBS, which promoted tight junction formation and the functional differentiation of NHU cells (3), but appeared to maintain NRU cells in a more proliferative and less differentiated state. This implies that there are fundamental differences in the signals regulating proliferation and differentiation between rat and human urothelial cells.

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Appendix F

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LONG-TERM CULTURE OF PORCINE BLADDER EPITHELIAL CELLS

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SUMMARY

Epithelial cells from normal pig bladders proliferated when cocultured with lethally irradiated feeder cells of the LA7 rat mammary tumor line. When the bladder cells and feeders were plated together at a confluent density, the bladder cells proliferated as the feeder cells died, resulting in a confluent culture of bladder cells. The bladder cells were successfully subcultured by plating with freshly irradiated LA7 feeder cells. In this way, bladder cells from five pigs were carried to confluency in passages 1, 4, 7, 7, and 13, amounting to at least 6, 18, 24, 26, and 45 doublings in culture, respectively, and none showed signs of slowed proliferation at the time of culture termination. Fibroblasts never became a prominent feature of these cultures, and their frequency was determined to be about 26 fibroblasts per 10^6 cells in passage 9. Pig bladder cells in 0.5% serum doubled in number in slightly over 3 d, whereas cells in 5.0% serum doubled in about 6 d. In fresh medium without feeder cells only minimal proliferation of bladder cells occurred. In LA7 conditioned medium the bladder cell numbers decreased, leading to the conclusion that the stimulus from LA7 cells is mechanically or physically transmitted. The bladder cells reacted with antibodies to keratins 7 and 18 but not to keratin 14 or vimentin. Tight junctions, visualized with an antibody to the ZO1 protein, connected all the cells to their neighbors. Most cells in passage 9 carried the diploid chromosome number of 38.

Key words: juxtacrine; cell proliferation; feeder cells; culture method; transitional epithelial cells; bladder.

Cultures of animal urothelial cells provide useful material for study, especially when human tissue is not available. Cultures of dog (Bonar et al., 1977), rabbit (Atala et al., 1992, 1993; Truschel et al., 1999), rodent (Noguchi et al., 1990; De Boer et al., 1994; Sterle, 1996, and several others), and pig (Guhe and Follmann, 1994; Fujiyama et al., 1995) bladder epithelial cultures have provided valuable information, especially with regard to the roles of growth factors and growth substrates on cell proliferation, differentiation, and senescence. Here, we report a unique culture system for pig bladder epithelial cells (PBEC), in which the growth stimuli are transmitted, not by factors added externally to the medium but by physical contact with neighboring cells of a different type. Bladder cells have achieved at least 45 doublings in number without any obvious signs of senescence. This culture system provides a model for juxtacrine growth stimulation of bladder cells and a method by which many cells can be generated for physiological studies.

Porcine bladder tissue was harvested from anesthetized minipigs (Stanek et al., 2001). The epithelial cells were cultured in a 1:1 combination of Dulbecco modified Eagle medium and Ham's F12 medium (Hazleton Biologics, Lenexa, KS), supplemented with 10 μg/ml insulin, 5 μg/ml transferrin, 0.5 or 5% fetal bovine serum (Sterile Systems, Logan, UT) or newborn calf serum (Sigma Chemical Co., St. Louis, MO), a supplement of trace minerals (Hammond et al., 1984), and antibiotics.

PBEC proliferated in culture when they were plated with lethally irradiated (6 Gy) feeder cells of the LA7 rat mammary tumor line (Dulbecco, 1979) at a confluent density. The PBEC proliferated as the LA7 cells slowly died, and eventually PBEC covered the entire surface of the flask. At this time about half of the area of a confluent flask was covered with PBEC in a single layer and the other half with multilayered cells. Although later passages of cells were routinely cultured on tissue culture plastic, the success of the primary cultures was improved if the PBEC were plated on collagen I–coated plastic. PBEC grown with feeder cells were subcultured by trypsinization of a confluent culture of PBEC, and dilutions of these, usually at 1:10, were plated with freshly irradiated feeder cells at a confluent density into a new culture vessel. In this way, cultures from five pigs were carried to confluency in passages 1, 4, 7, 7, and 13, amounting to at least 6, 18, 24, 26, and 45 doublings in culture, respectively. None of these cultures showed signs of slowed growth or senescence at the time of culture termination. For example, the culture in passage 13 became confluent after 12 d from a dilution of 1:10, about the same as for cells in earlier passages.

The doubling time of cells cultured in this way was a little over 3 d when the serum concentration of the medium was 0.5%, in contrast to a doubling time of about 6 d in medium with 5.0% serum (Fig. 1). PBEC plated without feeders multiplied only minimally.

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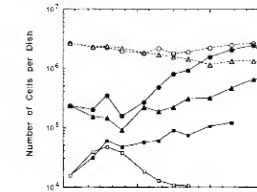

Fig. 1. Proliferation of PBEC. PBEC were dislodged from a confluent culture by trypsin-EDTA dissociation and plated at 1×10^5 PBEC per 45-mm petri dish either with or without feeders. On the d of the count the cells on the dish were dislodged by trypsin-EDTA and pelted through a 25-gauge needle to break the cell clumps into a single cell suspension. An aliquot of the suspension was diluted and counted on a ZBI Coulter counter (Coulter Electronics, Hialeah, FL) to determine the total cell numbers. The remaining cells were incubated with an HLG-conjugated mouse anti-rat MHC class I antibody (Pharmingen, San Diego, CA) at a dilution of 1:4. After incubation at 4°C for 1.5 h, the cells were preserved in 1% paraformaldehyde in PBS at 4°C until the d of analysis on a Becton-Dickinson FACScan (San Jose, CA). The Cellquest software program sorted 20,000 cells per sample according to fluorescence intensity. (a) Total number of recultured PBEC and LA7 feeders in medium with 0.5% serum. (●) number of PBEC cells in coculture with LA7 cells in 0.5% serum. (○) total number of recultured PBEC and LA7 feeders in medium with 5.0% serum. (▲) number of PBEC cells in coculture with LA7 cells in 5% serum. (■) number of PBEC cultured alone with fresh medium. (□) number of PBEC cultured alone with 1:2 fresh + 1:2 LA7-conditioned medium. EDTA, ethylenediaminetetraacetic acid; HRP, fluorescein isothiocyanate; MHC, major histocompatibility; cells; PBEC, pig bladder epithelial cell; PBS, phosphate-buffered saline.

mostly within the first few d. Thereafter, the numbers of PBEC in fresh medium with 0.5% serum increased slightly, but if plated in a mixture of fresh and LA7-conditioned medium, their numbers declined (Fig. 1). Most PBEC, if plated with feeders, survived the subculturing process, whereas PBEC plated alone had a plating efficiency about one-tenth of that of cells plated with feeders (Fig. 1). The morphology of PBEC also differed according to culture conditions. PBEC surrounded by LA7 feeders formed patches of closely packed cells with cobblestone morphology, whereas PBEC plated alone were more spread and varied in their sizes.

Confluence at culture initiation was essential not only for proliferation of PBEC, but also for inhibition of fibroblast growth. Fibroblasts were sometimes visible in the primary culture, but the frequency of these stems declined with each passage so that around passage 4 such stems were not detectable. An estimate of the number of fibroblasts in PBEC cultures carried to passage 9 with feeders was made by plating cells at low density in 5.0% serum and without feeders, conditions that favor fibroblast growth. After a few wk, the number of swirls and streaks characteristic of fibroblast growth were counted and calculated to represent about 2% fibroblasts per 10^6 cells.

PBEL produced keratin 18, associated with simple epithelia, and keratin 7 (Fig. 2), specific for transitional epithelial cells. In contrast, no keratin 14 or vimentin (Fig. 2) was detected within these cells. Tight junctions, visualized with an antibody to the ZO1 protein, joined PBEC at all their borders with each other and with the feeder cells (Fig. 3), confirming what the presence of domes suggests, namely, that these bladder cell cultures form an ionic barrier with transporting functions. Ten of twelve PBEC in passage 9 contained 38 chromosomes (Fig. 4), the diploid complement for pig, one cell contained 76 chromosomes, and another contained 74.

The special feature of the cell culture system described here is that the growth stimuli are transmitted in a direct physical way to the PBEC by the feeder cells. In these experiments, feeder-conditioned medium did not stimulate PBEC proliferation (Fig. 1). It is possible that the feeders mechanically trip a proliferation switch on neighboring PBEC. This juxtacrine mode has become much more appreciated in several epithelial systems (Wen et al., 2000) and has been fairly well studied for the epidermal growth factor receptor (EGFR) and its ligands (Shi et al., 2000). We know that feeder LA7 cells secrete transforming growth factor α (TGFα) into the medium (unpublished data) and that the secreted, membrane-bound TGFα is a ligand for the EGFR (Shi et al., 2000). Another component of stimulation in our culture system may involve the induction of more EGFR on the PBEC. Nguyen et al. (1990) have demonstrated that the mechanical stretching of human bladder cells in culture increases EGFR messenger ribonucleic acid synthesis by about a factor of 10. PBEC in our cultures, attached as they are by tight junctions to the feeder cells, could be mechanically stretched as various feeders die from radiation, and all the other cells in the monolayer are drawn together to maintain the integrity of the epithelial cell sheet. Thus, increased numbers of EGFR on the PBEC would be available to the TGFα lodged in the LA7 cell membranes.

Fibroblast feeders have often been used to promote the proliferation of epithelial cells in culture. Their mode of action differs from that of the epithelial feeders described here in that they secrete growth-stimulating factors into the medium. Fibroblast-conditioned medium has been shown to stimulate both human and porcine bladder epithelial cells in culture (Shaack et al., 2001), whereas conditioned medium from the LA7 epithelial feeders described here does not. Fibroblasts have also been shown to exert an influence on bladder epithelial cells by mechanical means (Tuiyama et al., 1995), although the mode is different from that exerted in the epithelial feeders provided here.

Gabe and Fellmann (1992) reported culturing porcine bladder cells in a serum-free medium, the most important growth-stimulating constituents of which were transferrin, insulin, EGF, and hydrocortisone. These cells could be carried for 5–6 passages within 12 wk, and the cells retained many of the characteristics of differentiated cells, such as tight junctions and microvilli. The cells slowly senesced, as determined by increased cell doubling times and increased fraction of multinucleated cells to 2.80%. The number of cell doublings undergone was not determinable from their data. In contrast to their culture system, ours maintained PBEC for at least 13 passages and 45 doublings without a decrease in growth rate.

Irradiated LA7 cells were originally used as feeder cells for the culture of primary mouse mammary epithelial cells (Ehninger et al., 1984), and the juxtacrine component of growth stimulation was described in these cultures (Fidman, 1992). Later, it was discovered

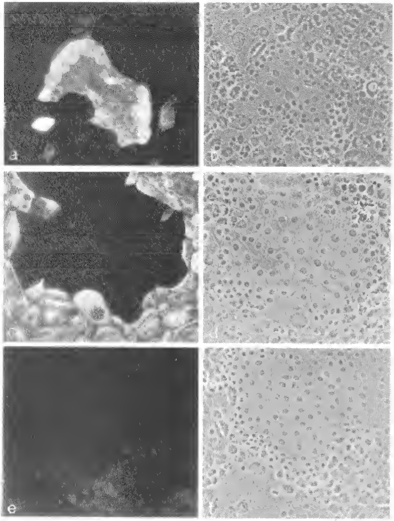

FIG. 2. Keratin 7 and vimentin in PBEC. Cultures were fixed in 1% paraformaldehyde or methanol at −20° C for 20–30 min and stored in PBS containing sodium azide at 4° C until the day of immunocytochemical staining. The cells were permeabilized with 0.5% Triton-x 100 in Hank's BSS for 20 min and incubated with the primary antibody for 1.5 h. The anti-keratin 7 monoclonal antibody (Chemicon International, Temecula, CA) was diluted by 1:200; anti-vimentin mouse ascites fluid (Sigma) was diluted by 1:50. After rinsing, an FITC-conjugated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) or an FITC-conjugated goat anti-rabbit IgG (Sigma), both diluted 1:100 with PBS, was applied to the cells, which were then incubated at 37° C for ~1 h. After rinsing, the coverslip was mounted over a solution of 2.5% 1,4 diazobicyclo[2.2.2]octane (Fluka Chemika, Ronkonkoma, NY) as a fluorescence saver and 90% glycerol in PBS. Cells were photographed on an inverted Nikon Diaphot microscope equipped with epifluorescence. (a) Fluorescent image of a PBEC colony and surrounding feeder LA7 cells incubated with an antibody to keratin 7. (b) Phase contrast image of cells in field a. (c) Fluorescent image of PBEC colony, in the center, and feeder cells, on the periphery, incubated with an antibody to vimentin. (d) Phase contrast image of cells in field c. (e) Fluorescent image of a field of cells incubated with a preimmune control serum. (f) Phase contrast image of cells in field e. PBEC colony in f covers the center to upper and right edges of the photograph. Magnification, ×130. BSS, basic salt solution; FITC, fluorescein isothiocyanate; PBEC, pig bladder epithelial cell; PBS, phosphate-buffered saline.

that LA7 cells also stimulate the long-term proliferation of rat mammary (Ehmann et al., 1991), mouse thymic (Ehmann et al., 1980), and human bladder epithelial cells (Ehmann and Terris, 2002). However, LA7 cells do not stimulate the multiplication of all epithelial cell types, including, most notably, human mammary cells (unpublished observations). Many epithelial tissues have been shown to respond to local rather than blood-borne factors, and the success of epithelial feeder cells probably depends on their ability to mimic the stimuli of cells found in the normal in vivo environment of the epithelial cells of interest. The ability of feeder cells to stimulate unrelated epithelia probably depends on the compatibility of their cell connection molecules and growth factor receptor pairs. It was undoubtedly an accident of evolution that LA7 rat mammary epithelial cells are similar enough to PBEC to promote proliferation of the latter. We would expect, however, from this point of view, that a pig bladder epithelial cell line would make an excellent feeder for normal primary pig bladder cells.

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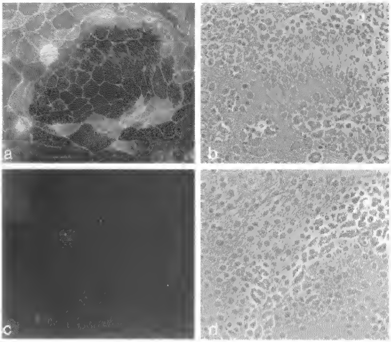

FIG. 3. Tight junctions in PBEC. (a) Fluorescent image of a colony of PBEC and surrounding feeder LA7 cells incubated with a polyclonal antiserum to the ZO1 protein of tight junctions, diluted 1:50 (Zymed Laboratories, San Francisco, CA). (b) Phase contrast image of cells in a. (c) PBEC and feeder LA7 cells incubated with a preimmune control serum. (d) Phase contrast image of field of cells in c. Two PBEC colonies, one on the *upper left* and one on the *lower right*, are divided by a streak of feeder cells. Magnification, ×135. PBEC, pig bladder epithelial cell.

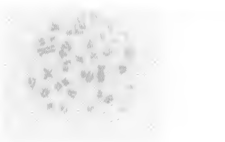

FIG. 4. Chromosomes of a pig bladder epithelial cell with a diploid complement of 38. After 2 wk in culture in passage 9, PBEC were arrested in metaphase for 6.5 h by 2 × 10[illegible] M colcemid (GIBCO, Santa Clara, CA). The cells were removed from the flask by trypsin-EDTA treatment, made hypotonic with 0.075 M KCl for 30 min, and fixed with 3:1 methanol–acetic acid. Cells were dropped onto humidified slides, stained with Giemsa, and viewed with a ×63 objective lens. Magnification, ×630. EDTA, ethylenediaminetetraacetic acid; PBEC, pig bladder epithelial cell.

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Appendix G

EUROPEAN UROLOGY 54 (2008) 1423–1432

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Reconstructive Urology

Generation of a Functional, Differentiated Porcine Urothelial Tissue In Vitro

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Abstract

Background: The primary function of urothelium is to serve as a physical urinary barrier. This function is dependent on features expressed at the molecular level that are acquired during cytodifferentiation. Urothelial cells lose differentiated and functional characteristics when propagated in vitro.

Objective: To investigate methods of inducing molecular and functional differentiation of normal porcine urothelial (NPU) cells in vitro.

Design and Measurements: NPU cells were isolated from normal porcine bladders and propagated in a low-calcium keratinocyte serum-free medium. Effects of 5% fetal bovine serum (FBS) and exogenous calcium were investigated. Molecular differentiation was assessed by immunolabelling for urothelial differentiation-associated proteins (UPIIIa, CK20, ZO-1), and barrier function was assessed by measurement of transepithelial electrical resistance (TER).

Results: NPU cell cultures grew as monolayers in low-calcium, serum-free medium. Supplementation with 5% FBS and/or physiological calcium resulted in stratification into basal, intermediate, and superficial cell zones. Superficial cells were positive for UPIIIa, CK20, and ZO-1. TER measurement showed that NPU cells grown with FBS had significantly enhanced barrier function (6720 ohms·cm^2 ± 1312 SD) compared with cells grown without FBS (102 ohms·cm^2 ± 34 SD; $p < 0.001$).

Limitations: Importantly, our study demonstrates that expression of differentiation-associated immunohistochemical markers by cultured urothelial cells can be regarded as evidence of only morphological differentiation and does not represent a surrogate marker of function.

Conclusions: We have shown that normal porcine bladder urothelium has many cell biological properties equivalent to normal human urothelium, making it an excellent research substitute for difficult-to-obtain tissue. A differentiated, functional barrier urothelium has been produced from porcine bladder urothelial cells propagated in vitro and displays molecular and functional properties equivalent to native urothelium. This tissue has application in developing tissue-engineered bladders with urinary barrier properties and as a research tool for understanding the relationship between molecular and functional tissue differentiation.

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1. Introduction

The function of the urothelium as a urinary barrier is conserved in its differentiation programme, particularly in the molecular specialisation of the terminally differentiated superficial cells. This process is exemplified in the expression of uroplakin proteins in the apical membrane [1,2] and by selective claudin proteins in the intercellular tight junctions [3], which contribute to transcellular and paracellular urinary barrier functions, respectively. A tight urothelial barrier is critical to bladder function and metabolic homeostasis; therefore, the identification of these differentiation proteins in cultured urothelium is important, because they are often assumed to be surrogate markers for the presence of a functional, differentiated urothelial tissue.

Advances in urothelial cell culture techniques over the past two decades have enabled normal human urothelial (NHU) cells to be cultivated almost routinely in vitro, where they express a highly proliferative phenotype to generate large and potentially clinically relevant numbers of cells [4]. This proliferative phenotype is akin to the wound-healing phenotype typical of damaged urothelium in vivo [4,5]. The conundrum is that cultured NHU cells do not spontaneously form an organised, differentiated, and functional urothelium [4,6] and, until recently, it has been unclear whether the potential of the cells to form a functional tissue was compromised, perhaps irreversibly, by tissue isolation and propagation procedures. Understanding the regulatory basis of the proliferation versus differentiation paradigm represents one of the key challenges to the development of a tissue-engineered urothelium replacement.

Some progress has been made towards dissecting the molecular pathways underlying proliferation and differentiation. The proliferative phenotype of NHU cells has been shown to be driven by an autocrine loop regulated through the epidermal growth factor receptor (EGFR) [7–11]. Furthermore, the inhibition of this autocrine loop by EGFR or downstream pathway inhibitors is a permissive step for inducing late/terminal urothelial differentiation through activation of the nuclear hormone receptor, peroxisome proliferator-activated receptor gamma [3,12]. It has also been shown that by manipulation of the culture conditions, such as the inclusion of bovine serum, NHU cells propagated in vitro are able to form a urothelium-like tissue with barrier properties [13].

A potential use for urothelium propagated in vitro is in composite cystoplasty. Here, it is proposed that autologous urothelium engineered in vitro would replace the mucus-producing, absorptive epithelium of the bowel segment used for enhancement or substitution of the bladder in conventional enterocystoplasty, as bowel epithelium is structurally and physiologically unsuited to exposure to urine [14–17]. The clear advantage of the composite cystoplasty approach is that it requires only one tissue component to be engineered in vitro; namely, the urothelium. Proof of concept of composite cystoplasty has been demonstrated in the marsupial, where urothelial cell sheets propagated in vitro were placed onto a deepithelialised smooth muscle segment of uterine origin and incorporated into the bladder [18]. No shrinkage of the neobladders occurred, and the animals voided normally. However, the study highlighted the importance of the functional status of the implanted urothelium. Inflammation of the stroma, which was observed both in native and augmenting segments, was interpreted as possible evidence of stromal exposure to urine as a result of poor urinary barrier properties of the undifferentiated tissue-engineered urothelial sheet [18]. This problem could be avoided by incorporating a differentiated, functional urothelium into the engineered bladder, where it might be expected to establish immediate urinary barrier properties. It could be anticipated that this approach would circumvent the problems of using immature urothelial tissues that are dependent on unknown in vivo factors to establish an effective urinary barrier. The use of a functional urothelium could be expected to confer more immediate protection of the underlying stroma, thus reducing the risk of inflammatory changes.

Surgically, the pig is a useful model because of its anatomical similarity to man. However, the biological equivalence of porcine and human urothelium at a cellular level has not been adequately documented. This is an important consideration if the pig is to serve as a reliable surgical model for bladder tissue engineering. Potentially, this has important implications beyond tissue engineering, because abattoir-derived normal porcine bladder urothelial cells could provide a readily available research surrogate for human bladder urothelium, which can be difficult to procure in the clinical setting. Thus, the purpose of this study was to investigate the differentiation and the functional potential of cultured normal porcine urothelial (NPU) cells.

2. Methods

2.1. Tissues

Whole porcine bladders were retrieved from a local abattoir into transport medium [4] (Hanks' balanced salt solution

[HBSS; Gibco, Paisley, UK] containing 10 mmol/l HEPES, pH 7.6, 20 kallikrein-inhibiting units/ml aprotinin [Trasylol; Bayer Pharmaceuticals, Newbury, UK], 1 μg/ml amphotericin B [Fungitrone; Gibco], 100 U/ml penicillin, and 100 μg/ml streptomycin). The bladders were opened and the serosa removed. The remaining tissue, divided into 1-cm^2 pieces, was incubated in 0.5% (w/v) dispase II in Puck's solution A (Roche Applied Science, Lewes, UK) at 37 °C overnight to allow the urothelium to be separated from the basement membrane. The isolated NPU cells were used to initiate finite cell lines, as described previously [18].

Primary cell cultures were established on Primaria tissue culture flasks (Becton Dickinson, Cowley, UK) at a seeding density of 4×10^4 cells/cm^2 in antibiotic-free keratinocyte serum-free medium (KSFM) containing recombinant epidermal growth factor and bovine pituitary extract at the manufacturer's recommended concentrations (Invitrogen Ltd, Paisley, UK) and 50 ng/ml cholera toxin (Sigma-Aldrich, Poole, UK) to improve cell attachment (complete KSFM [KSFMc] [4]). Serial subculture (passage) was performed on just-confluent cultures, as described previously [4], and cells were reseeded at 4×10^4 cells/cm^2. The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 (v/v) in air.

2.2. *Induction of differentiation of porcine urothelium*

The method to switch NPU cells from a proliferative to differentiated phenotype was adapted from Cross et al [13]. Briefly, established NPU cell cultures at passages 1–5 were either maintained in KSFMc (control), or the medium was supplemented with 5% fetal bovine serum (FBS; Harlan Sera-Lab, Loughborough, UK). At confluence, control and FBS-treated cultures were harvested and counted; 1×10^5 cells were seeded in the appropriate medium onto Snapwell membranes (1.13 cm^2; Costar, High Wycombe, UK). In some experiments, the exogenous calcium concentration was adjusted to 2.5 mmol/l by addition of $CaCl_2$ (1 mol/l stock solution) to reflect the physiological concentration of calcium in porcine serum (on the basis of calcium concentrations of 0.09 mmol/l for KSFM and 3.5 mmol/l for FBS). Exogenous calcium was added 24 h after the cells were seeded onto the Snapwell membranes in all experiments except permeability studies and their associated TER assessment, when it was added 16 h prior to testing.

NPU cells were maintained as four experimental groups: group 1 in KSFMc 0.09 mmol/l Ca^{2+}; group 2 in KSFMc 2.5 mmol/l Ca^{2+}; group 3 in KSFMc 5% FBS 0.22 mmol/l Ca^{2+}; and group 4 in KSFMc 5% FBS 2.5 mmol/l Ca^{2+}. The medium of both apical and basal chambers was changed on alternate days. Electrophysiological testing, permeability, and histological studies were performed after 7 d of culturing. Studies were conducted on interexperimental replicates of ≥ 9 and on three independent porcine bladder cell lines.

2.3. *Immunohistology*

Samples of native porcine bladder were trimmed and placed into 0.5% (w/v) zinc acetate:0.05% (w/v) zinc chloride fixative for 24 h. The tissue was dehydrated through ethanol into xylene and embedded in paraffin wax.

For cultures, medium from the basal and apical chambers of Snapwell membranes was replaced with 2% (w/v) dispase II in phosphate-buffered saline and incubated at 37 °C until the urothelial sheet detached. The sheet was incubated in zinc fixative for 10 min prior to processing as above, and 5-μm sections were mounted onto SuperFrost Plus glass slides (VWR, St Helens, UK). Representative samples were dewaxed and stained with haematoxylin-eosin. The remaining sections were dewaxed prior to immunoperoxidase labelling, as described [12]. Table 1 shows the antibodies used.

2.4. *Scanning electron microscopy*

NPU cells were maintained in culture in standard or serum-supplemented media. At confluence, each culture was harvested and 1×10^5 cells were seeded in the appropriate medium onto Snapwell membranes. Sixteen h prior to fixation, the calcium ion concentration was increased to 2.5 mmol/l in half of the membranes through the addition of 1 mol/l $CaCl_2$, as described previously. For fixation of the specimens, the media in the apical and basal compartments of the Snapwell membranes were aspirated and the membrane inserts removed. The samples were submerged in 100 mmol/l phosphate buffer containing 4% paraformaldehyde and 2.5% glutaraldehyde at pH 7 for 24 h in ambient conditions. The cell sheets were dehydrated through graded ethanol solutions into hexamethyldisilazane for 45 min, air-dried, sputter-coated with gold/palladium, and imaged on a JEOL JSM 6490LV scanning electron microscope.

2.5. *Electrophysiology*

2.5.1. *Transepithelial electrical resistance (TER)*

Snapwell membranes were inserted into the vertical chamber of an Ussing chamber (Warner Instruments Ltd, Hamden, CT, USA) and bathed in a calibrated Krebs solution (118 mmol/l NaCl, 4.73 mmol/l KCl, 2.34 mmol/l KH_2PO_4, 24.9 mmol/l $NaHCO_3$, 1.27 mmol/l $CaCl_2{\cdot}2H_2O$, 5.55 mmol/l D-glucose) maintained at 37 °C and equilibrated with 95% O_2:5% CO_2.

Table 1 – List of antibodies and suppliers

Antigen	Antibody	Concentration or dilution	Supplier
CK7	OV TL 12/30	0.1 μg/ml	Novocastra, Newcastle, UK
CK20	Ks20.8	0.07 μg/ml	Novocastra, Newcastle, UK
UPIIIa	AU1	1 in 100	Progen, Heidelberg, Germany
AUM	Anti-AUM	1 in 2000	Gift from T.T. Sun, New York University Medical School, USA
ZO-1	1A12	5 μg/ml	Zymed, Paisley, UK

Agar bridge electrodes (3% agar in 3 mol/l KCl encapsulating Ag/AgCl voltage and Ag current electrodes) were placed on either side of the membrane, and the potential difference and voltage-clamped current were obtained with a multichannel voltage-current clamp (model VCC MC2, Physiological Instruments, San Diego, CA, USA). The resistance across the urothelial sheet was calculated with Ohm's Law ($R = V/I$) and was corrected to a membrane area of 1 cm^2. TER was assessed for urothelia of three different cell lines grown in triplicate in each of the four media conditions, with supplementation of calcium occurring 24 h after seeding (group 1, $n = 11$; group 2, $n = 10$; group 3, $n = 11$; group 4, $n = 11$).

The average TER of blank Snapwell membranes was 20 ohms cm^2 ($n = 3$) when the potential difference and clamped current were recorded immediately after the circuit was opened. When allowed to progress, the current was unstable and continued to rise, resulting in an eventual negligible resistance. TER of the membrane alone was therefore considered to be zero.

2.5.2. Permeability studies

Medium was aspirated from apical and basal Snapwell chambers. Then 0.5 ml of 4-RDs fluorescein isothiocyanate-dextran (FITC-dextran; 1 mg/ml) was added to the apical chamber and 1 ml tracer-free medium was added to the basal chamber. After 3 h, two 400-µl samples of the basal medium were collected and analysed by fluorimetry against a standard FITC-dextran concentration curve. Permeability (µg/cm^2/h) was calculated as the concentration of FITC-dextran that had diffused through each sheet. The same sheets also underwent TER testing, as described above, so that resistance and permeability could be compared directly. Urothelia of three different cell lines grown in triplicate in each of the four medium conditions were used, with supplementation of calcium occurring 18 h prior to testing ($n = 9$ for each group).

2.6. Statistical analysis

Urothelial transepithelial electrical resistance values were averaged within each of the four groups and compared by Dunn's multiple comparisons test (non-parametric analysis of variance) with the use of Instat software v.3. (www.GraphPad.com). When two groups were compared, a two-tailed unpaired Mann–Whitney test was used; p values less than 0.05 were considered statistically significant.

3. Results

3.1. Cell culture

Because of the non-sterile source of porcine bladder tissue, a proportion of primary cell cultures were contaminated and therefore discarded. All other NPU cell cultures were propagated and subcultured successfully until cell numbers sufficient for electrophysiological or histological analyses were reached (typically passages 3–5). The morphology and behaviour of the cells were similar when grown on Primaria and Snapwell substrates, with islands of well-defined epithelioid cells expanding concentrically and coalescing to form a confluent cell sheet (Fig. 1a). NPU cell cultures maintained in serum-supplemented medium underwent a marked change in morphology, losing their discrete cell borders and taking on a more homogeneous, sheetlike appearance (Fig. 1b). These changes were also observed by scanning electron microscopy (SEM), in which the cells exposed to FBS appeared significantly more uniform than those grown in serum-free conditions, with cell-cell borders difficult to identify in most of the areas viewed (arrowed; Fig. 2A–D).

3.2. Histology

Porcine bladder was composed of a three-layer arrangement of serosa, smooth muscle, and urothelium. Above the basement membrane, the urothelium was stratified with basal and intermediate cell layers and a single overlying layer of large superficial cells (Fig. 3A). Urothelial cell cultures on Snapwell membranes in KSFMc alone (group 1)

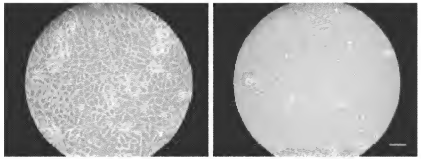

Fig. 1 – Phase-contrast microscopy of porcine urothelial cells in culture: (a) Undifferentiated cells in complete keratinocyte serum-free medium (KSFMc); (b) differentiated cells in KSFMc containing 5% fetal bovine serum and 2.5 mmol/l Ca^{2+}. Scale bar = 10 µm.

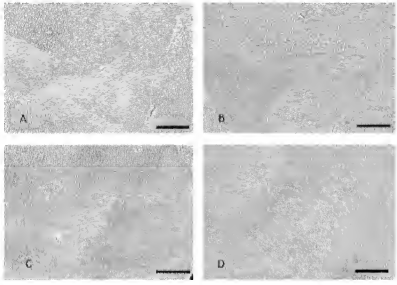

Fig. 2 – Scanning electron microscope micrographs of the surface of porcine urothelial cell cultures grown in complete keratinocyte serum-free medium (KSFMc) alone (Group 1; A), KSFMc containing 2.5 mmol/l Ca^{2+} (Group 2; B), KSFMc containing 5% FBS (group 3; C) and KSFMc containing 5% fetal bovine serum and 2.5 mmol/l Ca^{2+} (group 4; D). Scale bar = 10 μm.

fragmented into individual cells when treated with dispase II and could not be retrieved as an intact sheet. For this reason, no histological or immunohistochemical testing could be performed on these samples. Robust urothelial sheets were recovered from membranes in experimental groups 2–4. Haematoxylin–eosin staining showed urothelia from these groups to be stratified, with basal, intermediate, and large superficial-like cells evident (Fig. 3B and C).

3.3. Immunohistochemistry

CK 7, used as a positive control, labelled all three cell layers of urothelium in native tissue and was similarly positive in all cultured sheets, irrespective of the medium used (Fig. 4). Antibodies against CK20, UPIIIa, AUM, and ZO-1 were also positive in all cultured cells and showed the same distribution as in native urothelium, with CK20, UPIIIa, and AUM all localised to the luminal surface of superficial cells and ZO-1 appearing at superolateral "kissing points" of the same cells (Fig. 4).

3.4. Transepithelial electrical resistance

The mean TER for three independent cell lines in each of the culture conditions is shown in Fig. 5. Urothelial cell sheets grown in low-calcium, serum-supplemented medium (group 3) showed signifi-

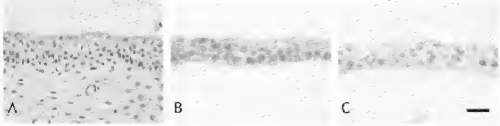

Fig. 3 – Haematoxylin–eosin staining in native porcine tissue (A) and in cultured urothelial cell sheets grown in complete keratinocyte serum-free medium (KSFMc) containing 2.5 mmol/l Ca^{2+} (B) or in KSFMc containing 5% fetal bovine serum and 2.5 mmol/l Ca^{2+} (C). Scale bar = 10 μm.

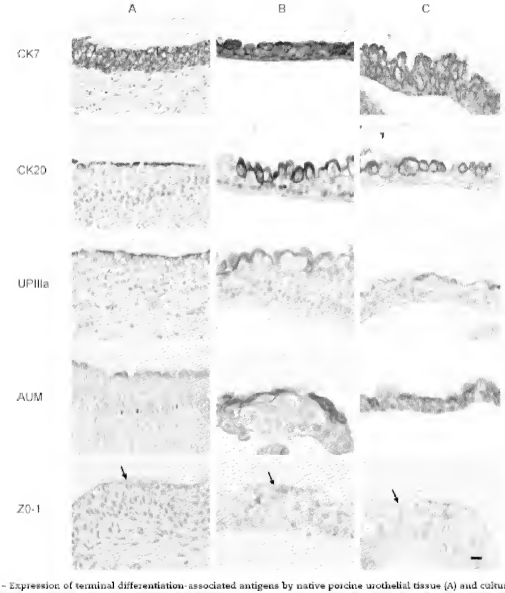

Fig. 4 – Expression of terminal differentiation-associated antigens by native porcine urothelial tissue (A) and cultured porcine urothelial cell sheets grown in complete keratinocyte serum-free medium (KSFMc) containing 2.5 mmol/l Ca^{2+} (B) or in KSFMc containing 5% fetal bovine serum and 2.5 mmol/l Ca^{2+} (C). Scale bar = 10 μm. Note that markers of terminal differentiation were expressed by cultured urothelial cell sheets irrespective of the medium in which they were grown.

cantly greater resistance than cultures grown in serum free medium, irrespective of the calcium concentration (groups 1 and 2; $p < 0.001$ and $p < 0.01$, respectively). Unexpectedly, serum-supplemented cultures maintained with physiological calcium concentrations (group 4) for long periods of time (6 d) showed a detrimental effect on TER, although the TER remained significantly higher compared with cultures grown in low-calcium, serum-free medium ($p < 0.05$).

Further investigation revealed that the detrimental effect of calcium on TER was concentration-dependent ($n = 3$, Fig. 6). The detrimental effect was also related to the length of time the sheets were exposed to an increased calcium concentration, with no effect seen when exposed for only 16 h.

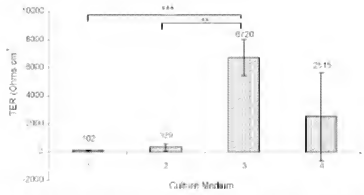

Fig. 5 – Mean transepithelial electrical resistance of three independent normal porcine urothelial cell lines, each grown in either complete keratinocyte serum-free medium (KSFMc) alone (group 1; n = 11), KSFMc containing 2.5 mmol/l Ca^{2+} (group 2; n = 10), KSFMc containing 5% FBS (group 3; n = 11) or KSFMc containing 5% FBS and 2.5 mmol/l Ca^{2+} (group 4; n = 11). Graphs display mean and standard deviation.

(5690 ohms cm^2 [group 3] versus 5683 ohms cm^2 [group 4], two-tailed $p = 0.94$ by Mann Whitney *U* test for five cell lines [n = 15]). This group included results found when the sheets used for permeability testing were assessed by TER (Fig. 7).

3.5. Permeability studies

Permeability of the sheets was inversely correlated with TER, with cell sheets in groups 1 and 2 exhibiting high permeability (1.91 ± 2.44 and 2.15 ± 2.53 μg/cm^2/h ± SD, respectively) and low TER, and those in groups 3 and 4 showing the opposite (−0.02 ± 0.04 and 0.12 ± 0.34 μg/cm^2/h ± SD, respectively; Fig. 7).

As discussed above, in group 4, addition of physiological levels of calcium to the sheets 16 h prior to testing negated any detrimental effect observed by a more prolonged exposure, and TER values were similar to those in group 3. Three replicates from group 2 (KSFMc + Ca) were discarded as tracer overspill into the basal compartment caused aberrant results.

4. Discussion

Native urothelium is a quiescent tissue with an extremely slow constitutive rate of cell turnover.

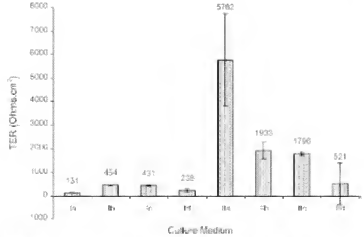

Fig. 6 – Mean transepithelial electrical resistance of a normal porcine urothelial cell line (n = 3) grown in either complete keratinocyte serum-free medium (KSFMc) (i) or KSFMc containing 5% fetal bovine serum (ii) with increasing final concentrations of calcium: (a) no added calcium, (b) final calcium concentration 1.5 mmol/l, (c) final calcium concentration 2 mmol/l, and (d) final calcium concentration 2.5 mmol/l. Graphs display mean and standard deviation.

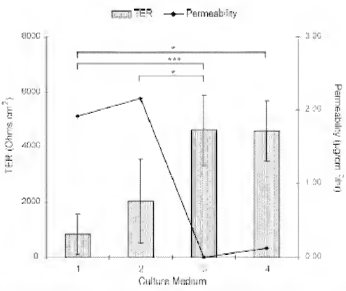

Fig. 7 – Mean transepithelial electrical resistance (TER) and permeability of three different normal porcine urothelial cell lines (n = 9), comparing proliferative and differentiating growth conditions and the effect of a physiological concentration of calcium added 16 h prior to testing. Error bars for TER represent standard deviation.

When injured, however, the urothelium displays a high regenerative capacity that allows the rapid re-establishment of an effective urinary barrier. In culture, NHU cells behave equivalently to NHU cells and grow as highly proliferative monolayers with a poorly differentiated phenotype and poor urinary barrier properties.

Despite the success of the first porcine model of composite cystoplasty as a functional augment, analysis at the cellular scale revealed stromal inflammation in both native and augmented segments. It was thought that this problem had probably occurred because the poor urinary barrier properties of the transplanted urothelial sheet allowed passage of urine to the suburothelial zone [18]; we hypothesised that this shortcoming may be related to the fact that proliferative, not differentiated, urothelium was used.

The ideal tissue-engineered urothelium must not only exhibit morphological and histological equivalence to the native structure, but also maintain a tight urinary barrier. The work presented here has shown the development of a differentiated and functional porcine urothelium from cells propagated in vitro that, when applied to the surgical model of composite cystoplasty, may encourage rapid formation of an effective urinary barrier.

We have shown previously that physiological calcium concentrations will induce stratification of NHU cell cultures without inducing differentiation [4], demonstrating that morphological stratification alone cannot predict the differentiated or functional status of the urothelium. By contrast, the presence of specific terminal differentiation-associated antigens, such as the uroplakins, might be expected to serve as surrogate markers for functional differentiation because their presence is recognised as critical for urinary barrier function [19]. However, the unexpected finding of this study was that all four differentiation markers (CK20, UPIIIa, AUM, and ZO-1) were expressed by NPU cell cultures irrespective of the medium used.

Only by assessing TER and permeability to FITC-dextran was the differential effect of serum evident on the development of a functional porcine urothelial barrier. Because TER and impermeability to dextran are contributed to primarily by the paracellular route, this finding implies that the effect of the serum was mediated through modulation of tight junction structure/function.

Because tight epithelia are considered to have a TER of >500 ohms cm^2 [20], NPU cell cultures propagated in serum-free medium with physiological calcium were, by definition, leaky, despite expressing a differentiated phenotype. By contrast, NPU cell cultures exposed to serum attained high TER and low permeability values, implying an effective paracellular barrier. TER values from native porcine urothelium were not compared with cultured

urothelial cells in this study; the difficulty in isolating a pure urothelial sheet from the bladder raises questions as to whether one can accurately compare like with like. However, in feline, guinea pig, rodent, and rabbit studies in which TER measurements of native urothelial tissue have been recorded, the values are of the same order of magnitude demonstrated in this study of cultured porcine urothelial cell sheets [21].

Unlike NHU cells [13], raising the calcium content of the serum-containing medium to a physiological concentration 24 h after seeding was detrimental to the TER, with an inverse relationship between TER and calcium concentration. Why porcine and human urothelial cells are different in this regard is at present unknown, but the finding highlights subtle differences between species that must be considered in development of preclinical models. Although not explaining the phenomenon, it is interesting to note that this difference was resolved when calcium supplementation to the serum-containing medium occurred 16 h prior to analysis, a time recognised as the minimum for inducing stratification in NHU cells [6]. In addition, immunohistochemical expression of the markers of differentiation remained unaffected by the timing of the addition of calcium.

5. Conclusions

We report the development of a porcine urothelial sheet generated in vitro with the morphological, histological, and functional properties of native urothelium. The intent is to use this tissue in a porcine surgical model of composite cystoplasty, where we propose that it will enable rapid establishment and maintenance of a functional urinary barrier in vivo without the need for further in vivo conditioning.

Our study raises an important issue because it indicates that expression of differentiation-associated immunohistochemical markers by cultured urothelial cells can be regarded only as evidence of morphological differentiation and cannot be used as a surrogate marker of function.

Finally, we propose that porcine bladder urothelium has many equivalent cell biological properties to human urothelium, making it an excellent research tool for difficult-to-source human urothelium. Taken together, the cell biological and anatomical similarities of porcine and human bladder tissues provide an important experimental model for developing tissue-engineering strategies, with the proviso that subtle differences are identified and considered during translation to the clinical setting.

Author contributions: Jennifer Southgate had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Turner, Subramaniam, Thomas, Southgate.
Acquisition of data: Turner.
Analysis and interpretation of data: Turner, Southgate.
Drafting of the manuscript: Turner, Southgate.
Critical revision of the manuscript for important intellectual content: Turner, Subramaniam, Thomas, Southgate.
Statistical analysis: Turner.
Obtaining funding: Turner, Thomas, Southgate.
Administrative, technical, or material support: None.
Supervision: Subramaniam, Thomas, Southgate.
Other (specify): None.

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